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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our operating hypothesis is that checkpoint suppressor 1 (CHES1)/FOXN3 is an androgen withdrawal-induced gene that promotes prostate cancer (CaP) resistance to apoptosis. The purposes of this research are two-fold. The first is to define the mechanisms of CHES1 gene expression regulation and function, particularly in mediating apoptosis resistance during androgen ablation. Secondly, the tools yielded from our functional studies will be utilized to test the efficacy of CHES1-silencing therapy (CST) in preventing castration-resistant prostate cancer (CRPC) and to develop a mechanism-based noninvasive imaging strategy for monitoring the success of CST. Several significant findings were made. We defined the mechanisms through which CHES1 coordinates anti-apoptotic pathways, specifically by enhanced PI3K/Akt activation and direct suppression of pro-apoptotic BNIP3 expression. Conversely, p53-mediated CHES1 down-regulation is required for genotoxic stress to trigger apoptosis. Another critical finding is that CHES1 directly interacts with the AR, which can figure prominently in influencing CaP progression to CRPC, since our findings demonstrated that while CHES1 potently inhibits transcriptional activity of wild-type AR, it enhanced the activity of constitutively-active AR splice variants associated with CRPC. Taken together, our findings provide strong support for exploiting CHES1 as a therapeutic target in that CHES1 antagonism would potentially lead to decreased anti-apoptotic PI3K-Akt signaling, reinstatement of pro-apoptotic gene expression (i.e., BNIP3), and reduced activity of oncogenic AR splice variants.

15. SUBJECT TERMS
Prostate cancer, forkhead, androgen receptor, p53, BNIP3, mTOR, hormonal therapy, apoptosis, chemotherapy, RNA interference, imaging

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INTRODUCTION

Androgen is a pivotal mediator of the growth, survival, and differentiation of prostate cancer (CaP) cells. Accordingly, androgen ablation is the first-line therapy for metastatic disease and dependably mediates disease regression. Unfortunately, this treatment is only palliative, as the disease typically recurs as castration-resistant prostate cancer (CRPC) approximately two years later and accounts for the 20% mortality rate due to this neoplasm. Therefore, defining the mechanisms underlying CaP survival during androgen withdrawal (AW) and the establishment of castration resistance are critical to enhancing our understanding of disease progression and the development of more efficacious therapies. We identified *FOXN3/CHES1* (*Checkpoint suppressor 1*) as a potential molecular mediator of CaP survival during androgen ablation. Our findings demonstrated that *CHES1* exhibits an AW-induced expression pattern and is an anti-apoptotic molecule that potentially acts via induction of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and/or down-regulation of the pro-apoptotic Bcl-2 family members *BNIP3* and *BAK1*. Importantly, antagonism of its function by RNA interference (RNAi)-mediated silencing resulted in apoptotic cell death of LNCaP cells selectively in the absence of androgen. That being said, ***the operating hypothesis of this work is that CHES1 is an AW-induced gene that functions to promote prostate cancer resistance to apoptosis and can be exploited as a therapeutic target.*** Therefore, there are two general purposes of this research. The first is to achieve a better understanding of mechanisms of *CHES1* gene expression regulation and function, particularly with respect to its role in mediating apoptosis resistance during androgen ablation. Secondly, the knowledge and tools yielded from our functional studies will be utilized to test the efficacy of *CHES1*-silencing therapy (CST) in preventing the emergence of CRPC and to develop a mechanism-based non-invasive imaging strategy for monitoring the success of the therapy.

BODY

This has been the second year of funding for this proposal and I am enthusiastically writing this report based upon the results from the work performed. Our team has been able to confirm preliminary findings described in the original grant proposal and more importantly, extend these along the lines of that described in our *Specific Aims* and the *Statement of Work*. These will be described in detail below and in the *Supporting Data* section of this report.

Development of tetracycline-inducible CHES1 expression model systems.

A required component for this task was to generate tetracycline (Tet)/doxycycline (Dox)-regulated LNCaP and CWR22Pc cell lines **(Task 5)**. As stated in the previous progress report, we first utilized a retroviral gene transfer approach with our pRevTRE-HA-CHES1 expression construct. Unfortunately, we could not detect expression of the CHES1 protein using either anti-HA or CHES1-specific antibodies. Therefore, as a second approach, we decided to switch expression systems to the Tet-inducible lentiviral vector pLVX-Tight-Puro (Clontech). For this, the *CHES1* CDS was first cloned into the pcDNA3.1-FLAG expression vector followed by automated sequencing for validation of sequence integrity and cloning of the CDS in-frame with the FLAG epitope tag. The FLAG-CHES1 cassette was then excised by HindIII/XhoI digestion, blunted, and ligated into NotI-digested pLVX-Tight-Puro, which had also been blunted and treated with calf intestinal phosphatase. Subsequently, Tet/Dox-inducible LNCaP-rtTA cells were infected with pLVX-Tight-Puro-FLAG-CHES1 lentivirus followed by selection with puromycin (1 µg/ml) to yield the new Tet-inducible CHES1 expression model, LNCaP-*tet*-FLAG-CHES1. In order to validate the model and address **Task 7**, a time-course experiment was performed to examine the kinetics and magnitude of Dox-inducible FLAG-CHES1 expression **(Fig. 1)**. As shown, FLAG-CHES1 expression was induced in a Dox-dependent manner and to

a physiological level. We are in the process of validating the CWR22P*c-tet*-FLAG-CHES1 model.

Determine the position of CHES1 within the molecular hierarchy of the apoptosis regulatory network. (Tasks 11, 14)

One of the primary goals of the proposal is to "*Define the mechanism(s) through which CHES1 regulates apoptosis and acts as a dominant mediator of prostate cancer survival during androgen ablation*" as stated in **Aim 1**. In addition to the induction of CHES1 expression, several key biochemical features of AW are the down-regulation of AR expression and transcriptional activity and up-regulation of PI3K-Akt signaling, with the latter being a dominant mediator survival in the absence of androgen. While these are clearly associated, a major task was to define a more mechanistic relationship between CHES1 and PI3K-Akt and the functional position of CHES1 within the molecular hierarchy of apoptosis regulation during androgen ablation **(Tasks 11, 14)**. We have made significant progress towards this.

The data implicate CHES1 as being an upstream mediator of these key events occurring in response to androgen deprivation. As shown in **Figure 1**, induction of CHES1 expression in LNCaP-tet-FLAG-CHES1 cells results in a marked reduction in AR expression and increase in Akt phosphorylation upon Ser473. In addition, BNIP3 expression was diminished, consistent with our hypothesis that CHES1 functions as a transcriptional repressor. While these are indeed exciting results, the exact mechanism as to how CHES1 mediates PI3K-Akt hyperactivation and AR down-regulation remained elusive. However, we believe we have made significant strides towards defining the mechanism. Specifically, ***our results demonstrate that mammalian target of rapamycin (mTOR), specifically mTOR complex 1 (mTORC1; containing raptor), is a principal downstream target of CHES1, which in turn coordinates PI3K-Akt hyperactivation and AR down-regulation.*** This is described below.

The first set of results demonstrates that mTORC1 functions as an integrator of AR and PI3K-Akt signaling during androgen withdrawal. Consistent with a previous report **(1)**, we find that androgen (R1881) strongly stimulated mTORC1 activity, but with slow kinetics **(Fig. 2, A and B)**. We extended these findings further to demonstrate that androgen has direct effects upon translation, as demonstrated by increased assembly of translational preinitiation complexes as measured by 7-methyl-GTP (m^7GTP) cap binding assays **(Fig. 2C)**. Androgen-induced mTORC1 activity, in turn, feeds back and inhibits (or limits) both AR expression and PI3K-Akt signaling. Conversely, we demonstrated that AW (2 and 5 days) resulted in a marked down-regulation in mTORC1 activity, as indicated by substantially reduced levels of Thr389-phosphorylated S6K1 **(Fig. 2A)**. This consequently led to de-repression of PI3K signaling and Akt hyperactivation, which was demonstrated by increased phospho-Akt(S473) levels **(Fig. 2A)**. The importance of mTORC1 inactivation to this was exemplified by the ability of the mTORC1 inhibitor rapamycin to mediate the same effects even in the presence of androgen. At the same time, AR levels also rapidly declined during AW, due to continued suppression of AR translation by residual androgen-independent (AI) mTOR activity coupled with the acute instability of the receptor in the absence of ligand. The importance of this "residual" mTOR signal during AW was underscored also by treatment with rapamycin, which completely abolished mTORC1 activity and restore AR expression and activity to levels equivalent to or greater than that observed in the presence of androgen **(Fig. 2A;** compare lanes labeled "*R*" to untreated "– " lanes**)**.

The next set of results demonstrates that androgen controls mTORC1 activation by regulating its association with the lysosome in response to amino acids. Recent advances revealed that amino acids stimulate mTORC1 (mTOR-raptor) by targeting it to the to the surface of lysosomes via interaction with Rag GTPases **(2,3)** and the trimeric Ragulator complex **(4)**,

which mediate association with it's activator, the G-protein Rheb. Proof-of-principle co-transfection/IP-western experiments conducted in 293T cells demonstrated that multimeric complexes of raptor and Rag heterodimers (A/B, A/C, and B/C) could easily be detected **(Fig. 3A)**. The same type of experiment was next performed in LNCaP cells in order to investigate the androgen-dependency of these interactions. As demonstrated in **Fig. 3B**, the interaction between raptor and Rag heterodimers, and therefore mTOR activity, is much more robust in the presence of androgen.

The third set of results demonstrated that CHES1 is a potent suppressor of mTORC1 activity by reducing it's targeting to the lysosomal surface. For this, we performed the same type of co-transfection/IP experiment in LNCaP cells that conditionally express CHES1 (LNCaP-*tet*-FLAG-CHES1). The results of this experiment showed that following induction of CHES1 expression (+*Dox*), there was significantly less raptor (*i.e.*, mTORC1) associated with Rag heterodimers **(Fig. 3C)**. In summary, the combined results suggest a mechanism in which AW leads to the up-regulation of CHES1 expression, which in turn decreases amino acid-mediated activation of mTORC1 as a result of decreased association of raptor with Rag heterodimers at the lysosomal surface. As a consequence of decreased mTORC1 activation, the PI3K/-Akt pathway is hyperactivated.

Another major advancement from this past year's work is the finding that CHES1 both physically interacts with and represses the activity of the AR. Based upon findings that other members of the forkhead family can directly interact with the AR **(5,6)**, we hypothesized that *CHES1*/*FOXN3* might also.   In order to investigate this, we performed standard co-transfection experiments with FLAG-tagged CHES1 and different forms of HA-tagged AR. Cell lysates were prepared, followed by immunoprecipitation with anti-HA and then immunoblot analysis for the presence of FLAG-CHES1 in the immune complexes. Four forms of the AR were used: full-length wild-type AR (ARwt), C-terminal truncation mutants containing only the N-transactivation domain (AR/NTD) or NTD plus nuclear-localization signal (AR/NTD-NLS), and the clinically-relevant splice variant AR3 **(7)**. As shown in **Figure 4A**, CHES1 interacts with full-length wild-type AR as well as all truncated variants tested. Interestingly, reporter assays conducted in LNCaP cells demonstrated that enforced expression of CHES1 represses AR transactivation of co-transfected pGL3-PSA(6.0)-luc **(Fig. 4B)**. The influence of CHES1 upon the transcriptional activity of the different AR variants was next investigated. Results from these experiments demonstrated that while CHES1 can repress activity of the full-length, wild-type AR, it has no inhibitory effect upon C-terminally truncated variants **(Fig. 4C)**. Taken together, this data suggest that down-regulation of AR during androgen ablation is due to several collaborating mechanisms: removal of ligand (DHT), mTORC1-mediated down-regulation of expression, and CHES1-mediated repression of its transcriptional activity. Moreover, truncated AR variants are not influenced by CHES1-mediated AR repression, thereby implicating this as a mechanism through which CaP cells can escape androgen ablation.

Define the regulation of CHES1 expression by p53-activating agents and if *CHES1* repression is a critical event in p53-mediated apoptosis. (Tasks 6, 21)

In addition to understanding how CHES1 engenders survival signaling, it is critical to define the upstream mechanisms that regulate its expression. Our operating hypothesis in this section of the proposal is that repression of CHES1 expression is a critical event in apoptosis mediated via p53-dependent mechanisms,, such as those stimulated in response to genotoxic chemotherapy. To investigate this, LNCaP cells were treated with mitomycin C (MMC) in dose-response and time-course experiments **(Fig. 5, A and B)**. MMC potently induced p53 with a concomitant decrease in CHES1 expression. Consistent with the survival-promoting mechanisms described for CHES1 above, CHES1 down-regulation was accompanied by a

reduction in phospho-Ak(S473) levels and increased BNIP3 expression. Importantly, this shift toward a pro-apoptotic manifested in the induction of apoptosis as indicated by PARP cleavage. The requirement for p53 as a mediator of CHES1 repression was investigated using an siRNA approach **(Task 21a)**. The results demonstrated that while treatment of LNCaP cells with Adriamycin led to p53 induction and CHES1 down-regulation, silencing of p53, markedly dampened the p53 response and stabilized CHES1 levels **(Fig. 5C)**, thereby demonstrating that p53 activation is required for CHES1 repression in response to chemotherapeutic agents.

Generate *CHES1* and *BNIP3* promoter firefly luciferase reporter constructs. (Task 13)

This task has been mostly completed. For the *CHES1* reporter, pGL3B-*CHES1*-RR1/3.5, a 3.5-kb region of the *CHES1* regulatory region (RR) spanning base positions -3,500 to +1 **(Fig. 6A)** was generated by high-fidelity PCR and then inserted into the pGL3-Basic firefly luciferase reporter vector. Similarly, pGL3B-*BNIP3*(4.0) was constructed by insertion of a 4.0-kb region of the *BNIP3* proximal enhancer/promoter spanning base positions -4,000 to +1, which contains 4 putative forkhead consensus binding sites **(Fig. 7A)**.

Define the mechanisms regulating *CHES1* gene expression. (Task 24)

Using the *CHES1*-RR1/3.5 reporter described above, we been able to better characterize the mechanisms regulating *CHES1* transcription. Using dual-luciferase reporter assays, we demonstrated that AW leads to 3.21-, 7.45-, and 16.71-fold induction in *CHES1* promoter activity by 48, 72, and 96 hours, respectively **(Fig. 6B)**. Although this is a direct response to the removal of androgen, it apparently is not mediated directly by the AR since the 3.5-kb regulatory region cloned does not contain any putative AR consensus binding sites **(Fig. 6A)**. Next, p53-mediated repression of CHES1 transcription was investigated. For this, reporter assays were performed by co-transfecting wild-type and mutant p53 expression constructs in combination with the *CHES1*-RR1/3.5 reporter. As shown in **Fig. 6C**, wild-type p53 potently represses *CHES1* promoter activity (*i.e.*, 77% of control) while the p53 loss-of-function and gain-of-function mutants either increase *CHES1*-RR1/3.5 activity (G245S) or have little or no effect.

Determine if CHES1 increases resistance to apoptosis by functioning as a transcriptional repressor of the pro-apoptotic gene *BNIP3*. (Task 16)

Regulation of BNIP3 promoter activity was investigated using the pGL3B-*BNIP3*(4.0) reporter described above. Reporter assays were performed with LNCaP cells treated with DHT, subjected to androgen withdrawal for 24-96 hours, and co-transfected with teo different CHES1 expression constructs. The results demonstrated that BNIP3 promoter activity was reduced 63% after 96 hours of AW **(Fig.7B)**. Importantly, demonstrated enforced expression of HA- or FLAG-tagged CHES1 mediated 73% and 89% reduction in activity.

Define the functional properties of CHES1 as an anti-apoptotic transcription factor. (Aim 2)

The LNCaP-*tet*-FLAG-CHES1 subline is also vital to accomplishing the goals of **Aims 2a and 2b**. One overall goal is to identify the direct and secondary target genes of CHES1 transcriptional regulation in a comprehensive and unbiased manner. For this, we performed RNA-Seq-based gene expression profiling **(Task 10)** of total RNA extracted from untreated cells and cells induced with Dox for 4 and 24 hours. In order to identify direct target genes of CHES1 and precisely map the location of CHES1 binding/recruitment, ChIP-Seq analysis was performed **(Task 16c)**. Importantly, we had originally proposed to use microarray-based expression profiling and standard chromatin immunoprecipitation (ChIP) assays for this. Instead, we decided to utilize next-generation sequencing (NGS), specifically RNA-Seq and

ChIP-Seq, respectively. In addition to NGS recently having become available at our Cancer Center's Genomics Shared Resource, the decision was based upon distinct advantages inherent in the technology, including its specificity and sensitivity. For ChIP analysis, in contrast to ChIP assays providing only information for pre-selected regions of the *BNIP3* locus, ChIP-Seq enables high-resolution mapping (*i.e.*, basepair level) of every CHES1 binding site in a completely unbiased manner. For both applications, single-read 36-basepair sequencing runs were performed and we are currently analyzing the data.

Establish LNCaP-*tet*-CHES1-Ri and corresponding vector control sublines as xenograft tumors and maintain by serial passage in athymic nude mice. (Task 9)

For this, xenograft tumors were established from the LNCaP-*tet*-CHES1-Ri-1 and -5 sublines (described in last year's progress report) and the corresponding vector control cell line by bilateral injection of a cell suspension of 1×10^7 cells in 50% Matrigel basement membrane matrix (1:1; v/v). The latency for tumor formation was approximately 7 weeks. These are currently at passage #2 and will next be used for the doxycycline (Dox) dose-optimization pilot studies described for **Task 12**.

Clinical significance of CHES1 expression

The studies proposed in this grant are designed to define the function of CHES1 and provide *proof-of-principle* for exploiting it as a therapeutic target. Although determination of the *CHES1* expression in clinical specimens was not proposed as a goal of this grant, we investigated the clinical significance of *CHES1* expression by performing a bioinformatics-based study of existing, publically available datasets derived from microarray gene expression profiling of clinical specimens. *CHES1* expression in clinical prostate cancer samples was surveyed in a microarray dataset **(8)** using the Oncomine web application (https://www.oncomine.org/resource/login.html). *CHES1* was down-regulated in 64% (16/25) of the samples relative to its expression in non-malignant prostatic epithelium **(Fig. 8A)**. Additionally, this analysis identified a 21-gene coexpression cluster composed of genes having ≥0.644 correlation with *CHES1* **(Fig. 8B)**.

KEY RESEARCH ACCOMPLISHMENTS
- Confirmed that CHES1 functions as a pivotal mediator of the survival response of prostate cancer cells to androgen ablation and defined this mechanism by demonstrating that mTORC1 is a principal downstream target of CHES1, which in turn coordinates PI3K-Akt hyperactivation and AR down-regulation.
- Discovered that CHES1 represses mTORC1 activation by inhibiting its association with Rag GTPases and targeting to the surface of the lysosome.
- Demonstrated that CHES1 directly interacts with the AR and represses transcriptional activity of wild-type AR, but not of truncated, constitutively-active variants associated with castration-resistant prostate cancer.
- Demonstrated that CHES1 down-regulation is required for chemotherapy-induced apoptosis and is mediated by p53-mediated transcriptional repression of CHES1 expression.
- Demonstrated that CHES1 directly represses promoter activity of the pro-apoptotic gene *BNIP3*.
- Gained insight into the clinical significance of CHES1 expression in that it is a gene normally expressed in non-malignant prostatic epithelium, but markedly decreased in primary prostate cancers.

REPORTABLE OUTCOMES

Abstracts
1. Xiang, N., Ali, H.M., Davis, R.R., Liu, S.Y., Boucher, D.L., Gregg, J.P., Kung, H.J., and Tepper, C.G. Potential role of *CHES1/FOXN3* as an anti-apoptotic regulator of prostate cancer response to androgen ablative and genotoxic therapies. Poster presented at: 15th Annual Cancer Research Symposium, October 28, 2010, UC Davis Cancer Center, Sacramento, CA
2. Tepper, C.G., Xiang, N., Ali, H.M., Gregg, J.P., Yang, J.K., Boucher, D.L, Wee, C.B., Webb, J.M., Liu, S.Y., de Vere White, R.W., Gregg, J.P., and Kung, H.J. Molecular Targeting of Prostate Cancer during Androgen Ablation: Inhibition of *CHES1/FOXN3*. Poster presented at: Innovative Minds in Prostate Cancer Today (IMPaCT) 2011 Conference, March 9-12, 2011, Hilton Orlando, Orlando, FL.

Presentations
1. Invited presentation, seminar entitled: "*Integrative genomics approaches to understanding prostate cancer progression*" November 12, 2010, National Tsing Hua University, Hsinchu, Taiwan, R.O.C.

Development of expression vectors and cell lines
During this past year of funding, we have generated a number of expression constructs and LNCaP and CWR22Pc sublines. These are listed below:
1. Constitutive *CHES1/FOXN3* expression construct with FLAG-epitope tag: pcDNA3.1-FLAG-*CHES1.*
2. Tet/Dox-inducible *CHES1* expression construct: pLVX-Tight-Puro-FLAG-*CHES1*.
3. Stable LNCaP sublines with Tet/Dox-inducible *CHES1/FOXN3* expression (LNCaP-*tet*-FLAG-CHES1) and corresponding vector-control cell line, LNCaP-*tet*-pLVX.
4. Stable LNCaP and 22R*v1* cell lines with constitutive expression of epitope-tagged mTOR, raptor, RagB-wt, RagB-GDP, RagB-GTP, RagD-wt, RagD-GDP, RagD-GTP.
5. *CHES1* Firefly luciferase reporter construct: pGL3B-*CHES1*-RR1/3.5
6. *BNIP3* Firefly luciferase reporter construct: pGL3B-*BNIP3*(4.0)
7. Stable CWR22Pc cell lines with constitutive expression of *CHES1* shRNAs: CWR22Pc-CHES1-Ri-1/3/7 and CWR22Pc-pSM2 vector control. **(Task 5)**

Funding applied for based on work supported by this award
1. Cancer Center Support Grant P30 (PI: de Vere White), NCI grant 2 P30 CA93373. The *CHES1/FOXN3* research supported by this grant contributed greatly to the Prostate Cancer Research Program component of the P30 renewal.
2. Prostate Cancer Program Project (P01) (NCI) Targeting novel androgen receptor-coactivator and miRNA pathways in prostate cancer. Project 3: *miR-124* and its role in prostate cancer tumorigenesis. Role: Co-I

Employment opportunities
Thankfully, the funding from this proposal has continued to provide employment and training for three individuals, in addition to myself. Dr. Nong Xiang is an extremely talented and tremendously industrious postdoctoral fellow/Associate Research Specialist and was hired specifically for this project. She has indeed made great strides and has been critical in driving this project forward. Mr. Hassen Ali was a valued research associate in my laboratory for the first 20 months of the award (April 15, 2009 – December 31, 2010) and left to pursue graduate

education. Beginning in January, 2011, this position has been filled by Mr. Shawn M. Purnell who recently graduated from UC Davis with a B.S. in Neuroscience, Physiology, and Behavior (NPB). Although Hassen was a key member of the team, we were fortunate to not have any interruptions in our experiments since Shawn had already been working in the lab as a volunteer for close to one year and had been fully trained in the necessary cellular, molecular, and biochemical techniques.

Educational outreach
    The research supported by this award also provided research opportunities and training to undergraduate students attending local community colleges, particularly American River College (ARC) in Sacramento, who generally cannot get this experience at their school. In conjunction with the American Medical Student Association (AMSA), I coordinated research internships for the newly developed AMSA ARC/UC Davis Cancer Center Community College Research Program." One of the interns from this program, Ms. Shanon Astey (sophomore) worked in my laboratory on the *CHES1/FOXN3* project and was very helpful in preparing plasmids and performing immunoblot analyses.

CONCLUSION
    The work of the past year provided several definitive findings, some of which have potential clinical significance. Our preliminary findings presented in the grant proposal demonstrated that *CHES1/FOXN3* was an androgen withdrawal (AW)-induced gene that mediated CaP survival by potentially functioning as an anti-apoptotic transcription factor. The results also demonstrated that heightened CHES1 expression was associated with key features of androgen ablation, specifically hyperactivation of the PI3K-Akt pathway and AR down-regulation. We now confirm that CHES1 is a pivotal mediator of this survival response and have elucidated the mechanism by demonstrating that mTORC1 is a principal downstream, functional target of CHES1 action, which in turn coordinates PI3K-Akt hyperactivation and AR down-regulation. A key element to this mechanism was the discovery that CHES1 represses mTORC1 activation in response to amino acid signaling by inhibiting its association with Rag GTPases and its targeting to the surface of the lysosome. As a consequence, the PI3K-Akt pathway is de-repressed and hyperactivated. We also demonstrated two novel mechanisms responsible for AW-induced AR down-regulation; one being due to residual mTORC1 activity driving down AR and the second one mediated through CHES1 directly interacting with the AR and suppressing it's activity. CHES1 further antagonizes apoptosis by directly repressing promoter activity and expression of the pro-apoptotic gene *BNIP3*. Conversely, we demonstrated that down-regulation of CHES1 is required for chemotherapy-induced apoptosis and is mediated by p53-mediated transcriptional repression of *CHES1*. These findings have several clinical implications. First, insight was gained into the clinical significance of *CHES1* expression by bioinformatics analysis of microarray datasets derived from clinical specimens. This revealed that *CHES1* is normally expressed in non-malignant prostatic epithelium, but markedly decreased in primary prostate cancers. Secondly, the CHES1-AR interaction can figure prominently in influencing CaP progression to CRPC since our findings demonstrated that although CHES1 potently inhibits transcriptional activity of wild-type AR, it enhanced the activity of constitutively-active AR splice variants associated with CRPC. Taken together, our findings provide strong support for exploiting CHES1 as a therapeutic target in that CHES1 antagonism would potentially lead to decreased anti-apoptotic PI3K-Akt signaling, reinstatement of pro-apoptotic gene expression (*i.e.*, BNIP3), and reduced activity of oncogenic AR splice variants.

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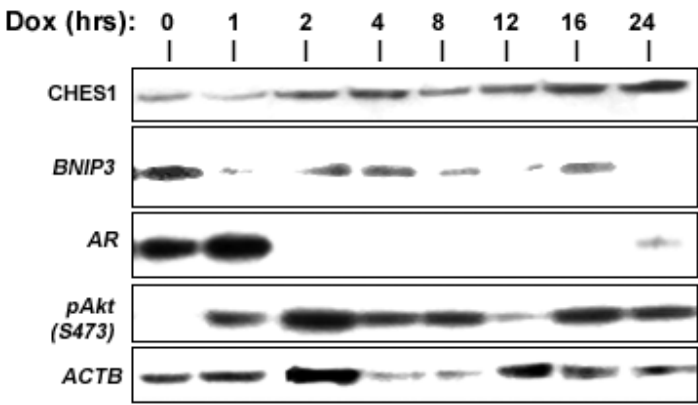
SUPPORTING DATA

Figure 1. CHES1 regulates key events associated with androgen ablation. LNCaP-*tet*-FLAG-CHES1 cells were left untreated (0 hr) or treated with Dox (100 µg/ml) for 1-24 hours. NP-40 lysates were prepared and immunoblot analysis performed to monitor the induction kinetics of CHES1 (anti-FLAG), the expression of BNIP3 and AR, and the level of S473-phosphorylated Akt. β-actin (ACTB) was used as loading control.
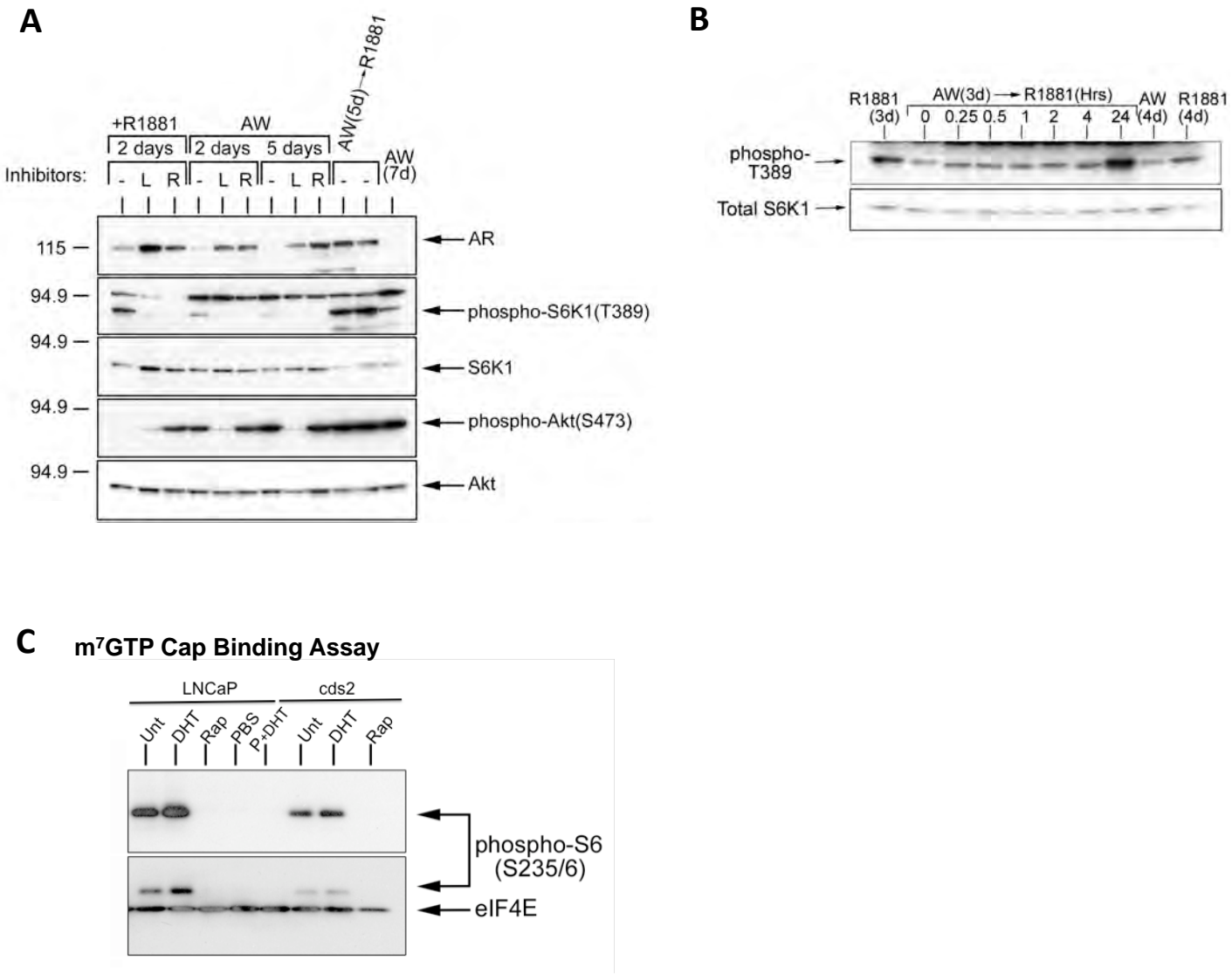
Figure 2. Androgen regulates mTOR activity. *A)* Total mTOR activity in LNCaP cells is the sum of androgen-dependent and-independent components. LNCaP cells were treated with synthetic androgen (*R1881*; 1 nM) or subjected to AW for 2, 5, and 7 days. After 5 days of androgen deprivation, two groups of cells were also treated with R1881 for an additional 1 and 2 days (*AW→R1881*). As indicated, cultures were also treated with the PI3K and mTOR inhibitors LY294002 (*L*; 10 µM) and rapamycin (*R*; 100 nM), respectively. In addition to the determination of AR and phospho-Akt levels, mTOR activation was monitored by immunoblot analysis for Thr389 phosphorylation of S6K1. *B)* Time-course experiments demonstrate that full reinstatement of phospho-S6K1(T389) levels proceeded with slow kinetics and required 24 hours. LNCaP cells were androgen-deprived for 3 days followed by stimulation with R1881 for the indicated time durations. Lysates were prepared and analyzed for levels of phospho(T389) and total S6K1. *C)* AR signaling promotes the assembly of the mTOR translational preinitiation complexes. m⁷GTP-binding assays were performed from androgen-dependent LNCaP and castration-resistant LNCaP-cds2 cells that were either androgen-deprived for 3 days or treated with DHT (1nM) or rapamycin (30 nM). Separate cultures were shifted into culture with PBS alone (*PBS*) or with DHT (1 nM) added (*P+DHT*).
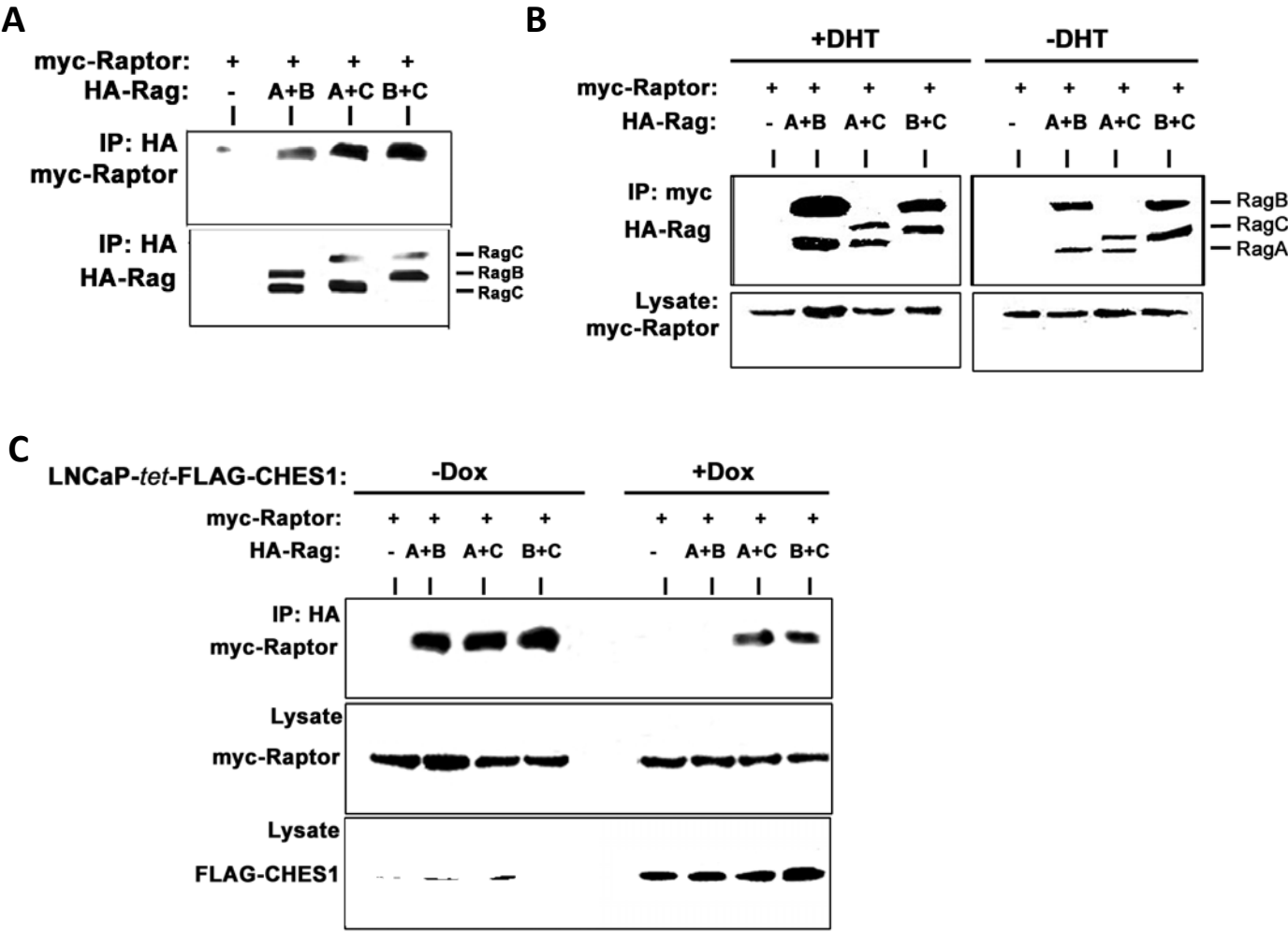
Figure 3. CHES1 mediates AW-induced down-regulation of mTOR by decreasing Rag-raptor recruitment to the lysosome. Co-IP analysis demonstrated that Rag heterodimers interact with mTOR complex 1 (mTORC1) via raptor in an androgen-dependent manner, which is inhibited by CHES1. The regulation of this interaction was investigated by co-transfection of expression vectors for the indicated HA-tagged Rag proteins in combination with myc-tagged raptor. *A)* Proof-of-principle IP-western studies for the Rag-raptor interaction were performed in 293T cells. The presence of raptor in HA-Rag immunoprecipitates was detected by blotting for anti-myc (*upper panel*) and confirmation of successful IP for Rag complex components with anti-HA (*lower panel*). *B)* Androgen regulates the interaction of raptor with Rag heterodimers. LNCaP cells were co-transfected as described above and cultured for 72 hours in the presence (*+DHT*) or absence (*-DHT*) of DHT. Co-IP analysis was performed by precipitation with anti-myc followed by immunoblotting for co-precipitated Rag complex components with anti-HA (*upper panel*) and control for raptor expression with anti-myc (*lower panel*). *C)* Enforced expression of CHES1 decreases Raptor-Rag interaction. LNCaP-*tet*-FLAG-CHES1 cells were transfected with myc-Raptor and HA-Rag expression vectors as described above followed by culturing in the absence (*-Dox*) or presence (*+Dox*) of doxycycline (100 µg/ml) to induce the expression of CHES1. Co-IP analysis was performed by IP with anti-HA and subsequent analysis for Raptor (anti-myc) in the immune complex (*upper panel*). Induction of CHES1 was validated by immunoblotting with anti-FLAG (*lower panel*).
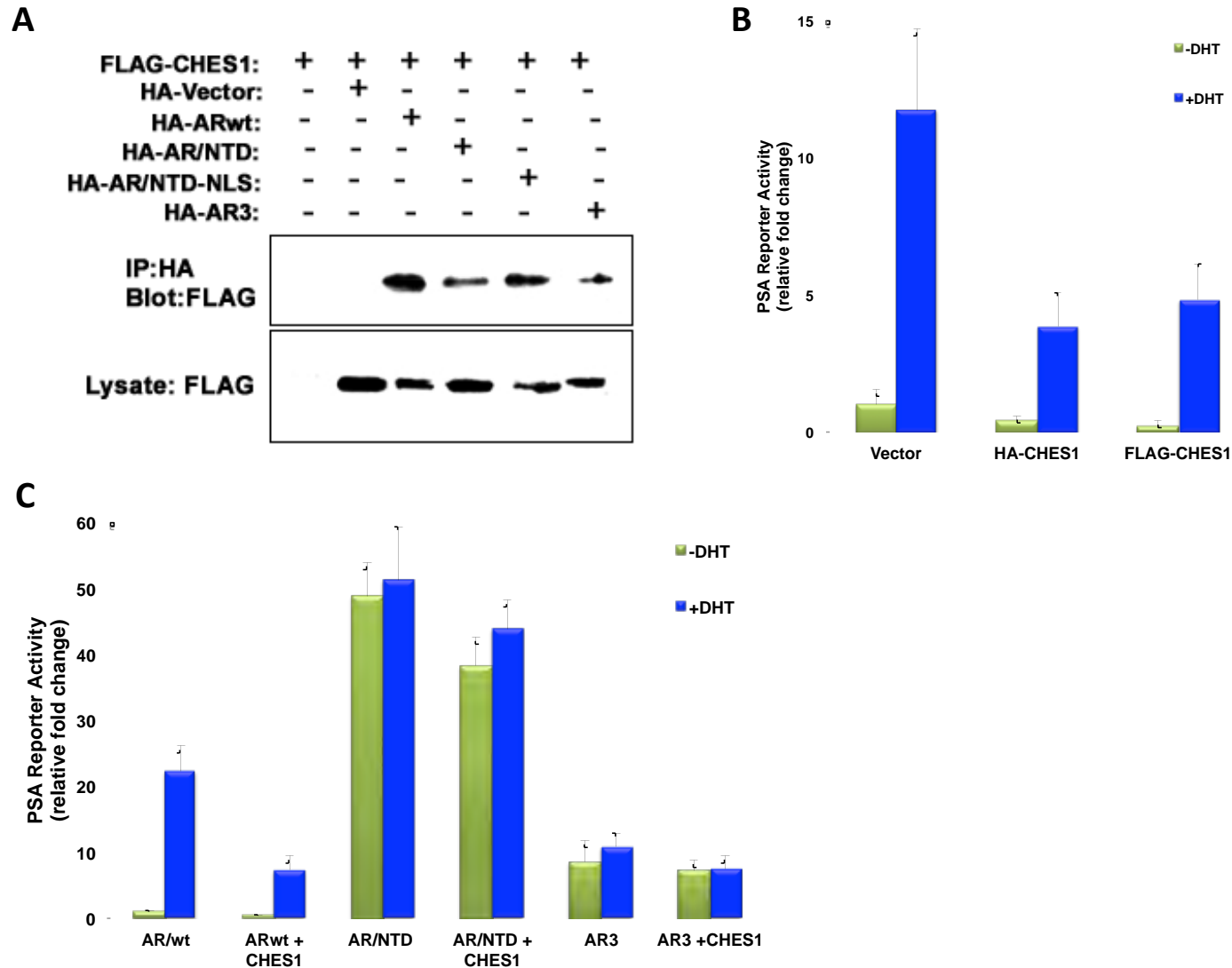
A

FLAG-CHES1:	+	+	+	+	+	+
HA-Vector:	-	+	-	-	-	-
HA-ARwt:	-	-	+	-	-	-
HA-AR/NTD:	-	-	-	+	-	-
HA-AR/NTD-NLS:	-	-	-	-	+	-
HA-AR3:	-	-	-	-	-	+

IP:HA
Blot:FLAG

Lysate: FLAG

B

C

Figure 4. CHES1 interacts with the AR and represses it's activity. **A)** IP-western analysis was performed to investigate the interaction between CHES1 and AR.   293T cells were co-transfected with expression vectors for FLAG epitope-tagged CHES1 in combination with HA epitope tagged AR.   Interaction with AR was examined for four forms: full-length wild-type AR (ARwt), C-terminal truncation mutants containing only the N-transactivation domain (AR/NTD) or NTD plus nuclear-localization signal (AR/NTD-NLS), and the clinically-relevant splice variant AR3.   Empty vector (HA-Vector) was included as a negative control. Samples were immunoprecipitated with anti-HA followed by blotting for FLAG.   **B)** LNCaP cells were co-transfected with the indicated CHES1 expression constructs (or empty vector as control) in combination with pGL3-PSA(6.0)-*luc* and pRL-SV40 reporter constructs. Cells were treated with DHT (1 nM) or left untreated (*-DHT*) for 24 hours and then harvested in Passive Lysis Buffer and dual-luciferase reporter (DLR) assays performed. In each sample, the PSA(6.0)-*luc* reporter activity (Firefly luciferase) was normalized to *Renilla* luciferase activity.   Results are presented as fold-change in PSA reporter activity relative to the Vector control (*-DHT*). **C)** Reporter assays were performed in 293T cells co-transfected with the indicated AR forms in combination with either the CHES1 expression construct or empty vector. Cells were then treated with DHT (1 nM) or vehicle control (*-DHT*) for 24 hours, followed by harvesting for DLR assays.   Results are presented as fold-change in PSA(6.0)-luc reporter activity relative to the AR/wt sample in the absence of androgen.
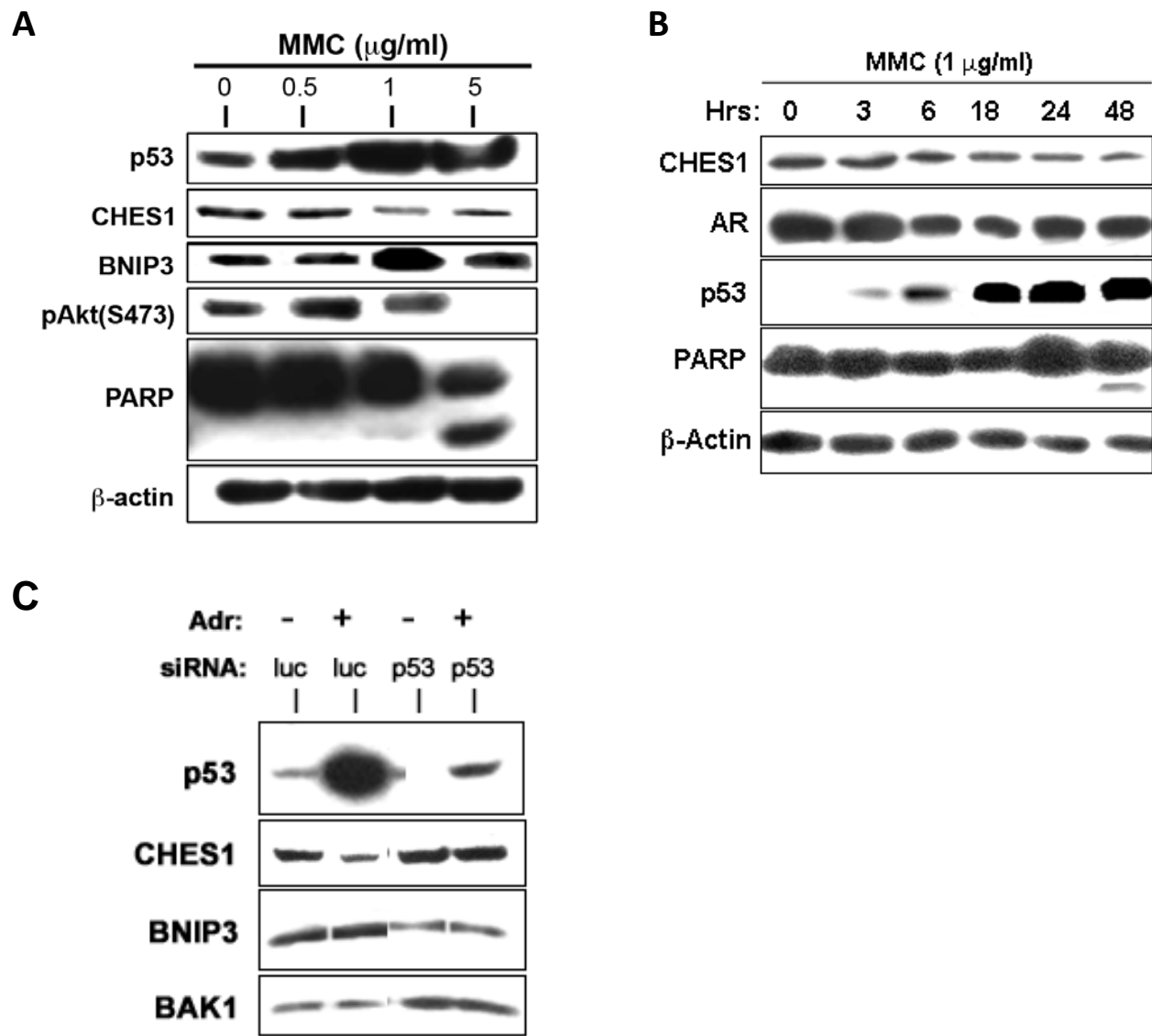
Figure 5. Chemotherapy-induced apoptosis potentially requires p53-mediated repression of *CHES1* expression.   The involvement of CHES1 in p53-mediated apoptosis triggered by chemotherapeutics was investigated for mitomycin C (MMC) and adriamycin (Adr). *A)* A dose-response experiment (0-5 µg/ml MMC) was performed in LNCaP cells followed by immunoblot analysis for the expression of p53, CHES1, and BNIP3, as well as Akt phosphorylation (S473), and the induction of apoptosis (PARP cleavage). Expression of β-actin was used as a loading control. *B)* A time-course experiment was conducted by treatment of LNCaP cells with MMC (1 µg/ml) for 0-48 hrs. Cells were harvested and processed for immunoblot analysis as described in "*A*" and analyzed for the indicated proteins. *C)* Attenuation of chemotherapy-induced p53 expression leads to maintenance of *CHES1* expression.   LNCaP cells were transfected with either siRNA targeting p53 or a control siRNA directed against *Firefly* luciferase (*luc*). After 48 hours, cells were then treated with adriamycin (Adr; 1 ug/ml; +) or PBS control (-) for 4 hours. Cell lysates were prepared and analyzed for expression of the indicated proteins.

A   *CHES1/FOXN3*

Chromosome: 14; **Location:** 14q31.3
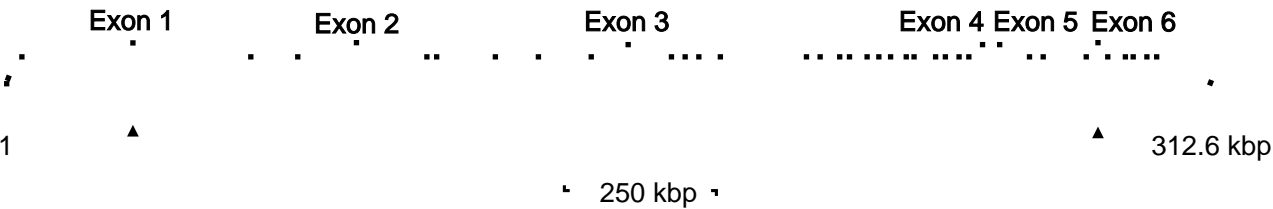
AR consensus: (A/G)(G/T)A(A/T)C(A/T/G)nnn(A/T/G)G(T/A/C)(T/A)(C/A)(T/G/C)
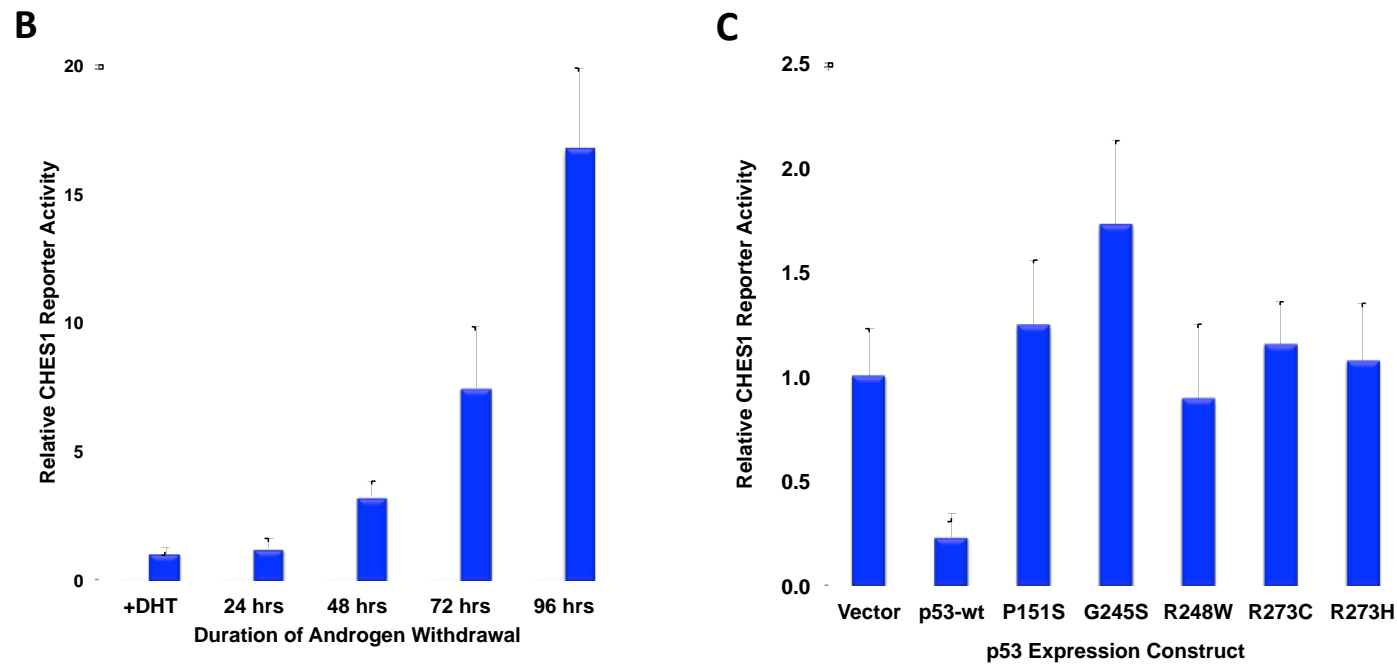

Figure 6. *CHES1* promoter activity is induced by androgen ablation and repressed by p53. *A)* Schematic of the *FOXN3/CHES1* genomic locus. The entire 312.6-kbp was screened for AR consensus binding sites (*tick marks*). Thirty-three were found throughout the gene, but none in the proximal enhancer/promoter. *B)* LNCaP cells were were co-transfected with the pGL3B-*CHES1*-RR1/3.5 reporter and pRL-SV40. The former contains 3.5-kb region of the *CHES1* regulatory region (RR) spanning base positions -3,500 to +1. Cells were then treated with DHT (1 nM) or subjected to androgen deprivation for 24-96 hours. At the indicated times, cells were harvested in Passive Lysis Buffer and dual-luciferase reporter (DLR) assays performed. In each sample, the *CHES1*-RR1/3.5 reporter activity (Firefly luciferase) was normalized to *Renilla* luciferase activity. Results are presented as fold-change in *CHES1* reporter activity relative to the DHT control. C) LNCaP cells were co-transfected as described above in combination with the indicated wild-type or mutant p53 expression constructs and harvested for DLR assays 72 hours later. Results are presented as fold-change in *CHES1* reporter activity relative to the Vector control.

A *BNIP3*

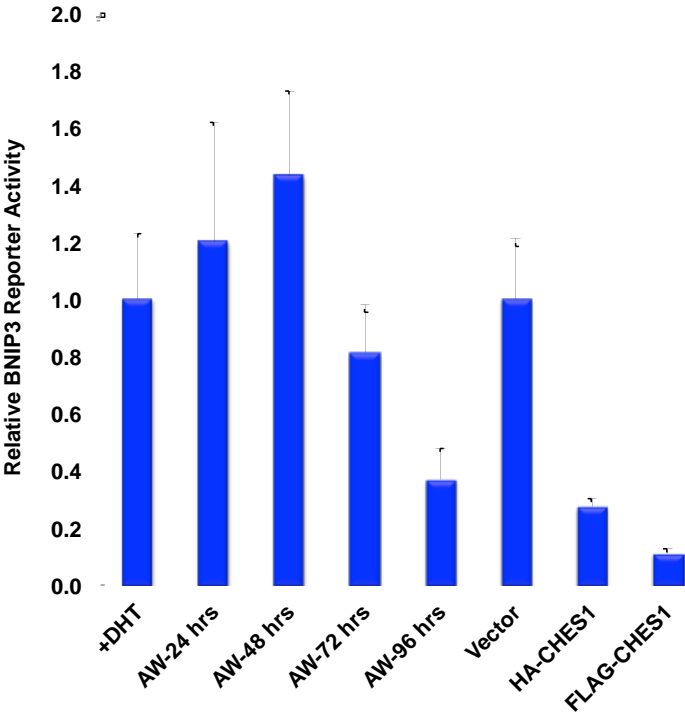**Chromosome:** 10; **Location:** 10q26.3
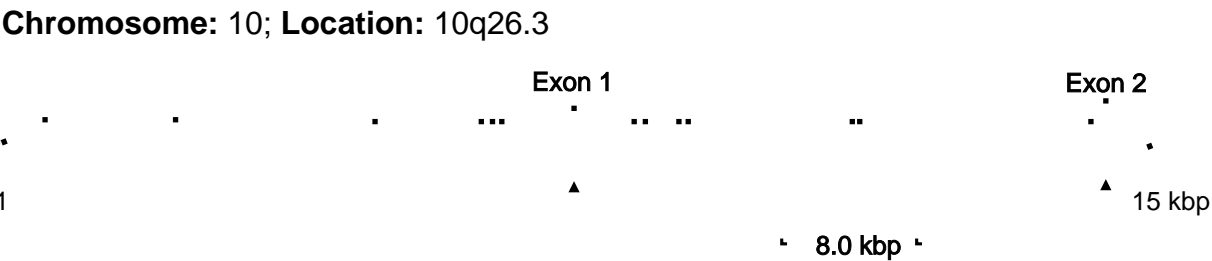
Figure 7. *BNIP3* promoter activity is decreased during androgen withdrawal and repressed by *CHES1*. **A)** Schematic of the *BNIP3* genomic locus. A 15-kbp region was screened for forkhead/FOX consensus binding sites (*tick marks*); five were found in the proximal enhancer/ promoter. **B)** LNCaP cells were were co-transfected with the pGL3B-*BNIP3*(4.0) reporter and pRL-SV40. The former contains a 4.0-kb region of the *BNIP3* proximal enhancer/promoter spanning base positions -4,000 to +1 and containing 4 potential forkhead binding sites. Cells were then treated with DHT (1 nM) or subjected to androgen withdrawal (AW) for 24-96 hours. In addition, cells were co-transfected with HA- and FLAG-CHES1 expression constructs and empty vector (*Vector*) as a control. At the indicated times, cells were harvested for dual-luciferase reporter (DLR) assays. In each sample, the *BNIP3*(4.0) reporter activity (*Firefly* luciferase) was normalized to *Renilla* luciferase activity. Results are presented as fold-change in *BNIP3* reporter activity relative to the DHT control or to that of empty vector.
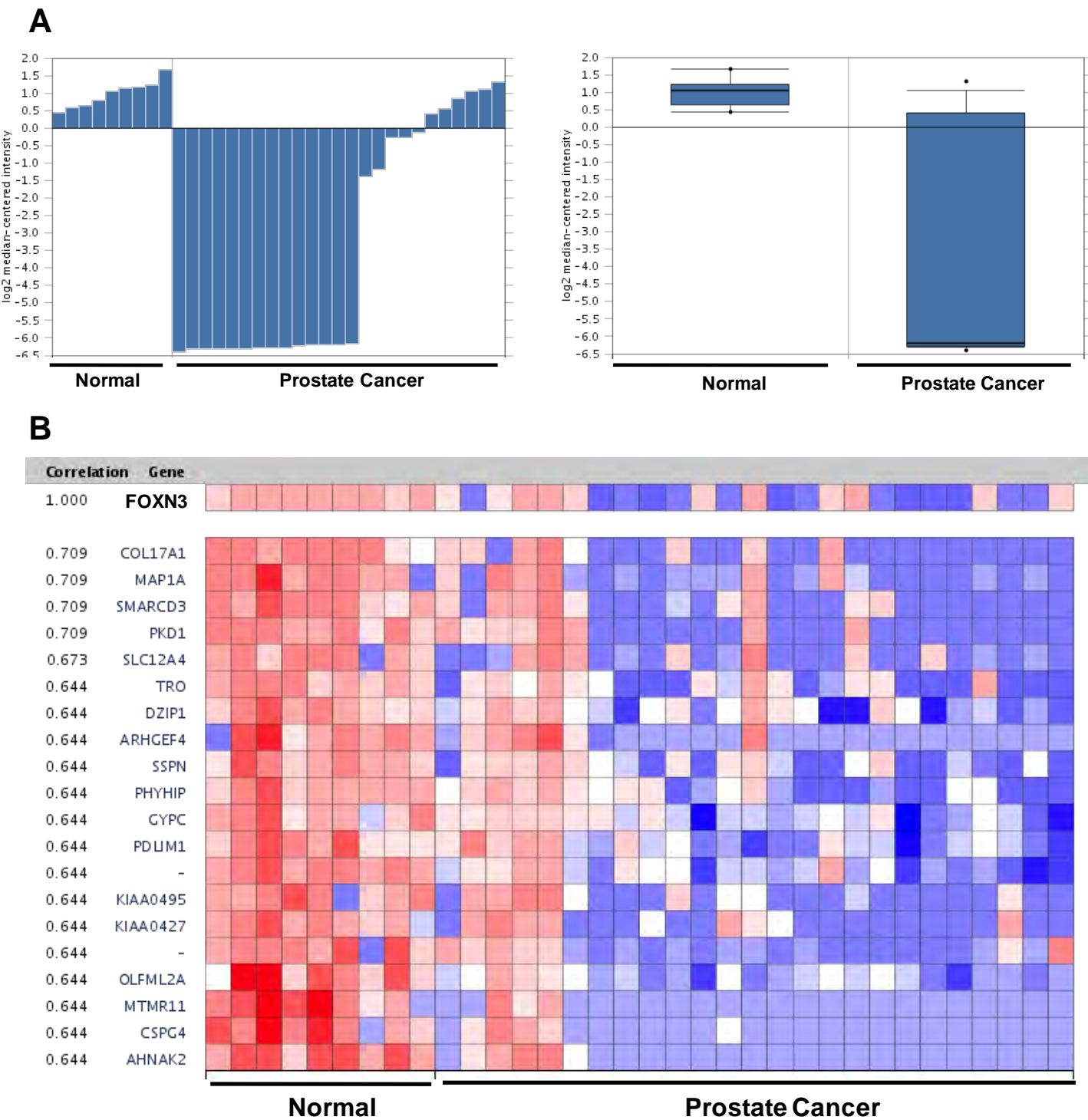
A

B

Figure 8. CHES1 expression is frequently down-regulated in prostate cancers. The expression of *CHES1* in clinical prostate cancer samples was surveyed in a microarray dataset (Welsh, JB *et al.* Cancer Res 61:5974-5978, 2001) using the Oncomine web application (https://www.oncomine.org/resource/login.html). *CHES1* was down-regulated in 64% (16/25) of the samples relative to its expression in non-malignant prostatic epithelium. *A)* *FOXN3*/*CHES1* expression level (signal intensity) in individual samples and as averaged expression in each group are depicted in the bar graph and box plot, respectively. *B)* The relative expression of *CHES1* and genes in its coexpression cluster is represented in the heat map.